Available online at www.sciencedirect.com

ScienceDirect

Developmental Biology 303 (2007) 311–324

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Cell cycling and differentiation do not require the retinoblastoma protein during early *Xenopus* development

Ruth A. Cosgrove [1], A. Philpott *

Department of Oncology, University of Cambridge, Hutchison/MRC Research Centre, Addenbrookes Hospital, Hills Road, Cambridge, CB2 0XZ, UK

Received for publication 29 March 2006; revised 3 November 2006; accepted 8 November 2006
Available online 11 November 2006

Abstract

The retinoblastoma protein (pRb) is a central regulator of the cell cycle, controlling passage through G1 phase. Moreover, pRb has also been shown to play a direct role in the differentiation of multiple tissues, including nerve and muscle. Rb null mice display embryonic lethality, although recent data have indicated that at least some of these defects are due to placental insufficiency. To investigate this further, we have examined the role of pRb in early development of the frog *Xenopus laevis*, which develops without the need for a placenta. Surprisingly, we see that loss of pXRb has no effect on either cell cycling or differentiation of neural or muscle tissue, while overexpression of pXRb similarly has no effects. We demonstrate that, in fact, pXRb is maintained in a hyperphosphorylated and therefore inactive state early in development. Therefore, Rb protein is not required for cell cycle control or differentiation in early embryos, indicating unusual control of these G1/G0 events at this developmental stage.

Keywords: Cell cycle; Differentiation; Retinoblastoma; *Xenopus*; Neural; Muscle

Introduction

The retinoblastoma protein (pRb) plays a central role in controlling passage through the cell cycle (Sherr, 1996) primarily by regulating the activity of E2F transcription factors (Dyson, 1998). At its simplest level, during early G1 phase, pRb is hypophosphorylated, allowing it to bind and inactivate E2Fs, thus preventing the expression of E2F target genes (Flemington et al., 1993), many of which are required for DNA synthesis (Muller et al., 2001). Late in G1, mitogenic signals such as growth factors promote the progressive hyperphosphorylation of pRb by cyclin D/CDK4 and 6 and cyclin E/CDK2, resulting in E2F release, activation of E2F target genes and progression into S phase (Sherr, 2000).

pRb also plays a direct role in tissue differentiation, which has been best described in myogenesis. pRb has two functions in terminal muscle differentiation: firstly, it is required to arrest the cell cycle by inhibiting E2F activity; secondly, its direct interaction with the muscle determination factor MyoD is required for the full transcriptional activity of the muscle differentiation factor MEF2. MEF2, in turn, induces expression of myosin heavy chain and muscle creatine kinase and the fully differentiated myocyte phenotype (Novitch et al., 1996; Novitch et al., 1999).

The ability of pRb to cause cell cycle arrest depends on its binding both E2Fs and DNA. However, its ability to promote differentiation by co-operation with transcription factors such as MyoD may be independent of E2F/DNA complex formation (Sellers et al., 1998). pRb plays a similar role in the differentiation of several other tissues, for example in adipogenesis (Chen et al., 1996), monocyte/macrophage differentiation (Chen et al., 1996), osteogenesis (Thomas et al., 2001) and epidermal differentiation (Nead et al., 1998; Ruiz et al., 2004). Thus, the promotion of terminal differentiation by pRb is not merely a consequence of its induction of cell cycle exit and, as a protein with two separable but complementary functions, pRb is well placed to co-ordinate these events.

* Corresponding author.
E-mail address: ap113@cam.ac.uk (A. Philpott).
[1] Current address: Babraham Institute, Babraham Research Campus, Cambridge, CB22 3AT, UK.

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.11.015

There are several lines of evidence which suggest that pRb performs a specific function in the differentiation of neural cells. Firstly, the most common cancer resulting from the loss of Rb is retinoblastoma, which originates from the neuroepithelial cells of the developing retina (Friend et al., 1986). This contrasts with the wide range of tumours that bear mutations in the genes of other members of the Rb pathway. Secondly, the level of Rb transcription is highest prior to and during the differentiation processes of neurogenesis, haematopoiesis, myogenesis, lens development and in the ganglion cell layer of the embryonic retina in mice (Jiang et al., 1997). In addition, there are distinct regulatory elements in the Rb promoter that are sufficient to direct expression of a fused transgene exclusively to the central and peripheral nervous systems (Jiang et al., 2001). The effect of overexpressing pRb in cultured cells supports the hypothesis that its activity is not only necessary but also sufficient to induce neural differentiation: CDK activities decline and pRb phosphorylation is consequently lost during experimentally induced differentiation of neuroblastoma cells, and overexpression of either the CDKI $p27^{Kip1}$ or of pRb itself is sufficient to induce neuronal differentiation in these cells (Kranenburg et al., 1995). These observations suggest that pRb may function as a determinant of neuronal differentiation. Recently, a role for pRb has been described as a coactivator of NeuroD1 transcriptional activity in mammalian cells (Batsche et al., 2005), although the factors responsible for the expression of Rb in the nervous system have not been identified.

A role for pRb in the development of several embryonic tissues was suggested from analysis of Rb null mice. Mice that express no functional pRb die between gestational days 14 and 15 exhibiting major developmental defects in the central nervous and haematopoietic systems, which show evidence of ectopic mitoses and massive cell death (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). However, intriguingly, much of the Rb null phenotype can be rescued by supplying Rb−/− embryos with wild-type placentae (Wu et al., 2003). Loss of pRb causes excessive proliferation of trophoblast cells and results in their failure to differentiate, leading to severe disruption of the structure of the placental labyrinth. This results in decreased vascularisation and significant impairment of placental transport function. Most of the neurological and erythroid abnormalities seen in Rb null embryos are therefore secondary to the effect of Rb loss on placental tissue (Wu et al., 2003).

Rb null embryos rescued by wild-type placentae survive until birth, when they die of skeletal muscle defects (de Bruin et al., 2003). These rescued embryos exhibit increased DNA replication and cell division in the CNS, similar to the phenotype of Rb knockout embryos. However, they do not show the widespread neuronal apoptosis which contributes to the lethality caused by Rb loss when the placenta is also Rb−/−. This indicates that the function of pRb in extra-embryonic lineages plays an important role in the suppression of apoptosis of neuronal cells, but not in the regulation of neuronal division and differentiation (de Bruin et al., 2003). However, to study the role of pRb in the earliest stages of vertebrate nerve and muscle differentiation, it would be helpful to turn to an organism in which embryos develop outside the mother, for instance those of the frog *Xenopus laevis.*

The *Xenopus* homologue of the retinoblastoma gene (*XRb*) shows 56% sequence homology with human and murine Rb at the amino acid level and is highly conserved in many functionally important regions (Destree et al., 1992). Maternal transcripts of XRb are highly represented in oocytes, while XRb protein levels increase tenfold between the unfertilised egg stage and stage 7.5 and then remain constant until stage 11.5. Zygotic transcription of the XRb gene is initiated at stage 13, and persists throughout early development and in all examined tissues of the adult frog, but expression levels vary greatly between tissues (Destree et al., 1992).

A study of *Xenopus* E2F revealed that the majority of E2F DNA binding activity exists in the free, uncomplexed form in the egg and throughout early embryogenesis (Philpott and Friend, 1994). Indeed, *Xenopus* E2F only begins to form complexes visible by gel retardation assay, and presumed to be with XRb, at neural plate stage 17 (Philpott and Friend, 1994). While more sensitive detection of E2F binding proteins revealed that XRb can bind E2F in the very early embryo, XRb is found only at a very low level at these early stages (Destree et al., 1992). Moreover, at this time only a small proportion of XRb binds E2F, leaving E2F predominantly uncomplexed until at least stage 35, although analysis of *Xenopus* tissue culture cells revealed significant E2F-XRb complexing by metamorphosis (Philpott and Friend, 1994).

Here, we have investigated the role of XRb in regulation of both cell cycling and differentiation during early development. XRb is expressed most highly in developing neural tissue of *Xenopus* embryos. However, surprisingly we find that modulation of the levels of XRb protein, by either depletion or overexpression, does not perturb cell cycle regulation, nor the differentiation of nerve or muscle tissue. These observations may be explained by the predominantly hyperphosphorylated, i.e., inactive status of pXRb that we detect throughout early *Xenopus* embryogenesis. Thus, pXRb is subject to developmental control, but is not essential for the early stages of development of *Xenopus*, perhaps representing fundamental differences in regulation of cell division and differentiation at early versus late developmental stages.

Results

Expression pattern of XRb in the early Xenopus embryo

It is becoming increasingly apparent that genes that regulate the cell cycle are not always expressed at equal levels in all proliferating cells, which may indicate additional functions in the development and differentiation of different tissues (Vernon and Philpott, 2003). To determine the developmental expression of XRb in the early *Xenopus* embryo, we performed whole mount in situ hybridisation to detect mRNA transcripts (Fig. 1). While XRb is barely detectable before neural plate stages (R. Cosgrove, A. Vernon, A. Philpott, unpublished data), expression becomes visible in the neural plate and anterior placodal regions including the eye

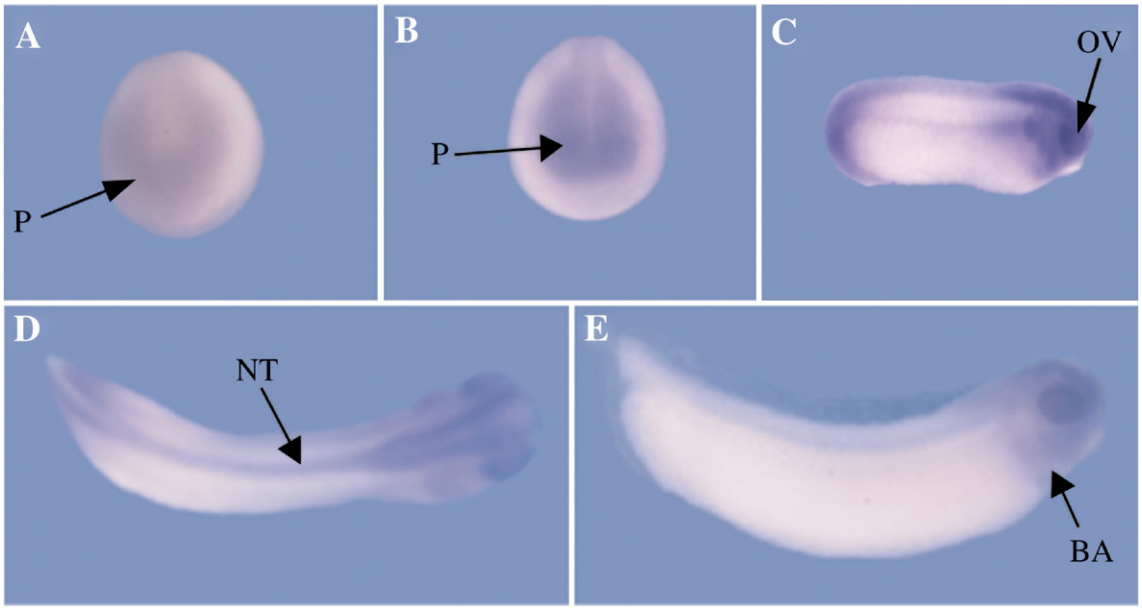

Fig. 1. Expression pattern of the *Xenopus* retinoblastoma gene. XRb expression was analysed by whole mount in situ hybridisation (stained in purple) in embryos at a range of stages. XRb is expressed in the anterior placodes (P) from stage 16 and increases by stage 19, especially in the optic vesicles (OV). Between stages 26 and 30, strong expression is seen in the optic vesicles, neural tube (NT) and branchial arches (BA). (A) Stage 16, anterior view; (B) stage 19, anterior view; (C) stage 26, lateral view; (D) stage 28, dorsal view; (E) stage 30, lateral view. Anteriors to the right (C, D, E).

field around stage 15 (Fig. 1A). By stage 19, XRb is strongly expressed in the anterior placodes (P) and optic vesicle (OV), as well as in the developing neural tube (NT) (Fig. 1B). As development progresses, staining intensifies in the eye, brain and neural tube, as well as in the branchial arches (BA) (Figs. 1C, D, E). Interestingly, while the role of pRb in the regulation of skeletal myogenesis has been well documented in mammalian cultured cell systems (Novitch et al., 1999; Sellers et al., 1998), XRb is not highly expressed in the myotome. Therefore, although XRb transcripts were detected throughout early *Xenopus* embryogenesis and in all adult tissues examined (Destree et al., 1992), its expression is greatly upregulated in developing neural tissues during the period when primary and secondary neurogenesis occurs (Hartenstein, 1989). This elevated neural expression is consistent with a conserved function of pRb in vertebrate neural tissues (Batsche et al., 2005; Jiang et al., 1997, 2001; Kranenburg et al., 1995), although its absence from the developing myotome is of note.

Depletion of pXRb from the early embryo

In order to investigate the effect of loss of XRb protein in developing *Xenopus* embryos, we microinjected an antisense morpholino oligonucleotide (Mo) targeted at the translational start site of XRb mRNA (XRb Mo), which should prevent translation of XRb protein (Heasman, 2002). To determine the effect on XRb protein expression, XRb Mo or a control morpholino (Con Mo) was injected into both blastomeres of two-cell stage embryos, and these were subsequently allowed to develop up to tailbud stage 22. Extracts were prepared from embryos at three stages and Western blotted for XRb protein (Fig. 2A). XRb Mo completely blocked translation of endogenous pXRb at stages 10, 15 and 22. In contrast, pXRb was still present after injection of Con Mo.

XRb protein is not essential for cell cycle progression during early embryogenesis

A wealth of evidence has implicated pRb in the regulation of the cell cycle (de Bruin et al., 2003; Sherr, 2000; Weinberg, 1995). Moreover, by the time of their death between gestational days 14 and 15, the phenotype of Rb null mice shows evidence of major cell cycle dysregulation, specifically ectopic mitoses and massive apoptosis, which causes developmental defects in the nervous and haematopoietic systems (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). However, more recently it has been established that some of these effects may result from placental defects in Rb null mice (de Bruin et al., 2003; Wu et al., 2003). As *Xenopus* embryos develop externally, we have investigated whether loss of XRb perturbs cell cycle regulation during early embryogenesis in the absence of a placental requirement. To this end, XRb protein was depleted by injecting the XRb Mo into one dorsal blastomere at the four-cell stage to target the morpholino to the developing nervous system where XRb is most highly expressed (Fig. 1). As the first plane of cleavage determines bilateral symmetry within the embryo, morpholino injected in this way is incorporated unilaterally within the neural tube, while the uninjected side of the embryo acts as an internal control. Embryos were allowed to develop to stage 21 before serial sectioning and immunostaining for phosphorylated histone H3 (pH3), a marker of mitotic cell proliferation (Saka and Smith, 2001), used to detect effects of pXRb depletion on cell cycling in the neural tube (Fig. 2B). Surprisingly, XRb Mo had no significant effect on cell proliferation when comparing the injected and uninjected sides

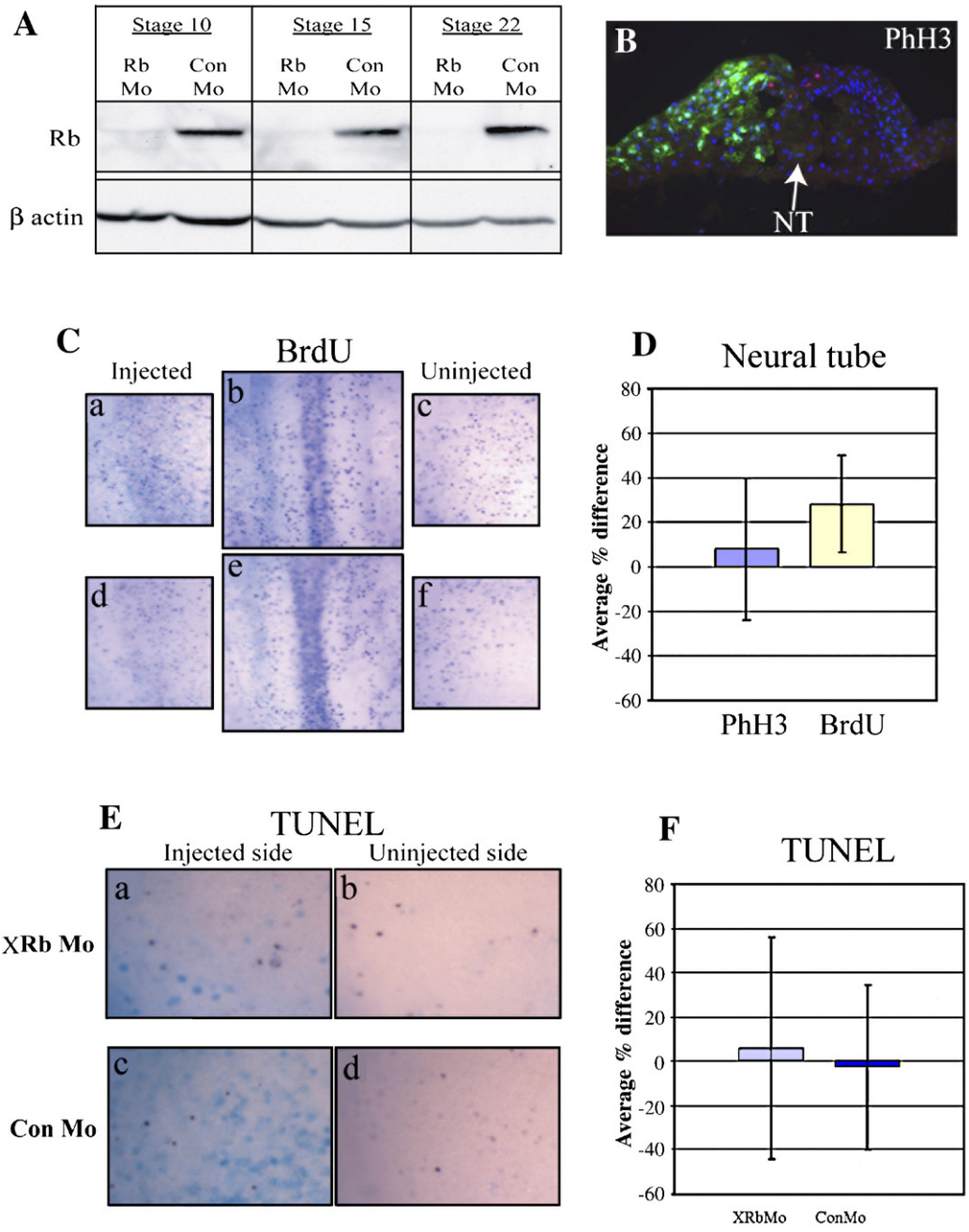

Fig. 2. XRb morpholino specifically depletes XRb protein but does not affect the number of cells in mitosis or S phase in the neural tube, or the number of cells undergoing apoptosis or S phase in the epidermis. (A) Embryos were injected with 20 ng XRb morpholino (Mo) or control Mo in both blastomeres at the two-cell stage. Extracts made from these embryos at stages 10, 15 and 22 were Western blotted and probed with anti-Rb antibody (XZ160). The same blot was stripped and probed with an anti-β-actin antibody to show equal loading. XRb Mo depletes XRb protein at all three stages, while control Mo does not affect its level. (B) Embryos were injected with 20 ng XRb Mo and β-galactosidase as a tracer (visible as green autofluorescence) in one dorsal cell at the four-cell stage. At stage 21, embryos were sectioned and immunostained using an anti-phospho histone H3 antibody to visualise mitotic cells (red fluorescence) in the neural tube (NT). Hoechst staining of nuclei (blue). (C) Embryos were injected in one cell at the two-cell stage with 20 ng XRb Mo (Ca–Cc) or control Mo (Cd–Cf) and β-galactosidase as a tracer (blue) and then injected with BrdU at stage 20. After 2 h they were fixed and epidermal cells in S phase were visualised by whole mount BrdU immunostaining (purple). There is no difference between the density of cells in S phase in the Mo-injected sides of the embryos (Ca and Cd) and their density in the uninjected sides of the same representative embryos (Cc and Cf). Dorsal views with injected sides on the left and uninjected sides on the right (Cb and Ce). (D) Embryos were injected with 20 ng XRb Mo, with or without subsequent BrdU injection (as in C), sectioned and immunostained with anti-phospho histone H3 or anti-BrdU antibody. The numbers of cells in mitosis or S phase in both injected and uninjected sides of the neural tubes were counted in serial sections ($n=6$ embryos for each marker, average of 41 sections per embryo). Average percentage differences between the sides are shown, with error bars showing the standard errors. pXRb depletion results in no significant change in the number of mitotic cells on the injected side of the neural tube compared to the uninjected side (average 8% increase, $p=0.2$), or in the number of cells in S phase (average 28% increase, $p=0.43$). (E) Embryos injected with 20 ng XRb Mo (Ea and Eb) or control Mo (Ec and Ed) with β-galactosidase as a tracer (blue) in one cell at the two-cell stage were fixed at stage 22. Apoptotic cells in the epidermis were visualised by whole mount TUNEL staining (purple). (F) The numbers of TUNEL-stained cells were counted in sample areas on both the Mo-injected and uninjected sides. Average percentage differences between the sides are shown, with error bars showing the standard errors. Neither XRb Mo ($p=0.44$; $n=10$) nor control Mo injection ($p=0.1$; $n=11$) affects the number of apoptotic cells in the epidermis (cf. injected sides, Ea and Ec, with uninjected sides, Eb and Ed).

of the neural tube (Fig. 2D; $n=6$ embryos, average 42 sections per embryo; $p=0.2$) and compared to the Con Mo-injected control (data not shown), indicating that pXRb is not essential for regulation of cell proliferation at this stage, even in the neural tube where it is most highly expressed.

XRb is most highly expressed in the nervous system, as detected by in situ hybridisation (Fig. 1), yet it may be expressed in other tissues at a lower level, and indeed can be detected in ectodermal explants (animal caps) that have differentiated into atypical epidermis, by RT-PCR (data not shown). While pRb is widely expressed in mice, it is not expressed at the same level in all cell types: for example, pRb protein levels are elevated in the brain and liver (Bernards et al., 1989; Szekely et al., 1992). Therefore, the effect of pXRb depletion on mitosis in the epidermis was also analysed by injection of XRb Mo (or Con Mo) into one cell of a two-cell embryo followed by phospho-histone H3 immunostaining. The numbers of mitotic cells in sample areas on the flank of injected and uninjected sides of a series of injected embryos were considerably higher than those seen in the neural tube and were counted. As expected, as pRb is only expressed endogenously at a low level in the epidermis, analysis showed that XRb depletion caused no significant change in the number of mitotic cells in the epidermis (data not shown; $p>0.05$, $n=19$).

It is possible that loss of XRb could perturb the cell cycle prior to mitosis and that this could subsequently result in apoptosis of these cells rather than a precocious mitotic entry. To investigate this, we injected XRb Mo or Con Mo into one cell of two-cell stage embryos. Embryos were fixed at early tailbud stage and the distribution of cells in S phase was investigated by staining for bromodeoxyuridine (BrdU) incorporation. Firstly, we analysed BrdU incorporation in the neural tube in embryos injected unilaterally with XRb Mo in sections (Fig. 2D; $n=6$ embryos, average 39 sections per embryo). We saw no significant difference in BrdU incorporation in the injected versus the uninjected side (Fig. 2D; $p=0.43$) or when compared to the Con Mo-injected control (data not shown).

Analysis of BrdU incorporation in the epidermis was also performed in either XRb Mo-injected embryos (Figs. 2Ca–Cc; $n=20$,) or Con Mo-injected embryos (Figs. 2Cd–Cf), and no significant difference was seen.

Similarly, the number of cells undergoing apoptosis on the injected versus the uninjected sides of the embryos was analysed by whole mount TUNEL staining. There was no significant effect on TUNEL staining after injection of either XRb Mo (Figs. 2Ea–b; $p=0.44$; $n=10$ embryos) or Con Mo (Figs. 2Ec–d, $p=0.1$, $n=11$ embryos) in the embryonic epidermis (Fig. 2F). Thus, severe reduction in the levels of XRb did not significantly affect cell cycle progression in either the neural tube, where it is expressed at high levels, or in the epidermis, where its expression is lower. Moreover, loss of XRb did not significantly increase apoptosis.

XRb protein is not essential for neural or muscle differentiation

Thus unexpectedly, we could not demonstrate a role for XRb protein in cell cycle regulation in the early *Xenopus* embryo. Nevertheless, XRb is highly expressed in neural tissue. Moreover, numerous papers have studied the potential role of pRb in the regulation of differentiation in a variety of tissues, including nerve and muscle. In particular, Rb knockout mice show severe neural defects (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992) and pRb has been demonstrated to play a direct role in neural differentiation in mice (Lee et al., 1994). However, the results from Rb knockout mice rescued with normal placentae shed some doubt on the absolute requirement for pRb in neurogenesis (de Bruin et al., 2003; Wu et al., 2003).

Thus, we investigated a role for XRb in development and differentiation of neural tissue in early *Xenopus* embryos in the absence of a placental requirement. To this end, embryos were injected in one blastomere at the two-cell stage with XRb Mo (or Con Mo), allowed to develop to stage 15 and their expression of a range of markers of neural differentiation and patterning analysed by whole mount in situ hybridisation (Fig. 3). The expression of the marker of neural determination NCAM (Balak et al., 1987), the marker of terminal neural differentiation neural β-tubulin (Richter et al., 1988) and the regional neural markers Engrailed (Eizema et al., 1994), Otx2 (Pannese et al., 1995), Pax6 (Hirsch and Harris, 1997) and Xbf1 (Bourguignon et al., 1998), were not affected by XRb protein depletion (Figs. 3A–F; $n=40$ per marker). Moreover, no effect was seen with Con Mo injection (data not shown). Neurite outgrowth in XRb morpholino-injected embryos at stage 28 was also examined by whole mount immunostaining with an anti-neural β-tubulin antibody. Neurites grew normally in both pXRb-depleted and control sides of the injected embryos (Fig. 3: compare G and H; $n=4$). These results indicate that XRb protein does not play an essential role in early stages of neuralisation, neural differentiation or patterning in *Xenopus* embryos.

A direct requirement for the Rb protein in the process of muscle differentiation, over and above its ability to regulate the cell cycle, has been demonstrated in cultured cell experiments (Novitch et al., 1996; Novitch et al., 1999). However, XRb is not expressed at a high level in the *Xenopus* myotome (Fig. 1). Nevertheless, the requirement for XRb protein in myogenesis was investigated by injecting XRb Mo into one blastomere at the two-cell stage. The expression of muscle actin, an early marker of muscle specification, and myosin heavy chain, a marker of terminal differentiation, were examined by whole mount in situ hybridisation at stage 15 (Figs. 3I, J). The expression patterns of these markers of muscle differentiation were not affected by pXRb protein depletion ($n=40$ per marker), indicating that pXRb is not required for muscle differentiation in *Xenopus* embryos. Moreover, somites form normally in XRb Mo-injected embryos, as visualised by β-gal-expressing nuclei aligning correctly in V-shaped muscle blocks (Figs. 3G, G′).

In summary, surprisingly, depletion of XRb protein does not have a significant effect on either cell cycle progression or differentiation of the *Xenopus* early embryonic nervous system or myotome. This may reflect the external development of *Xenopus* embryos and strengthens the hypothesis that many of the phenotypes seen in the Rb null mouse represent problems

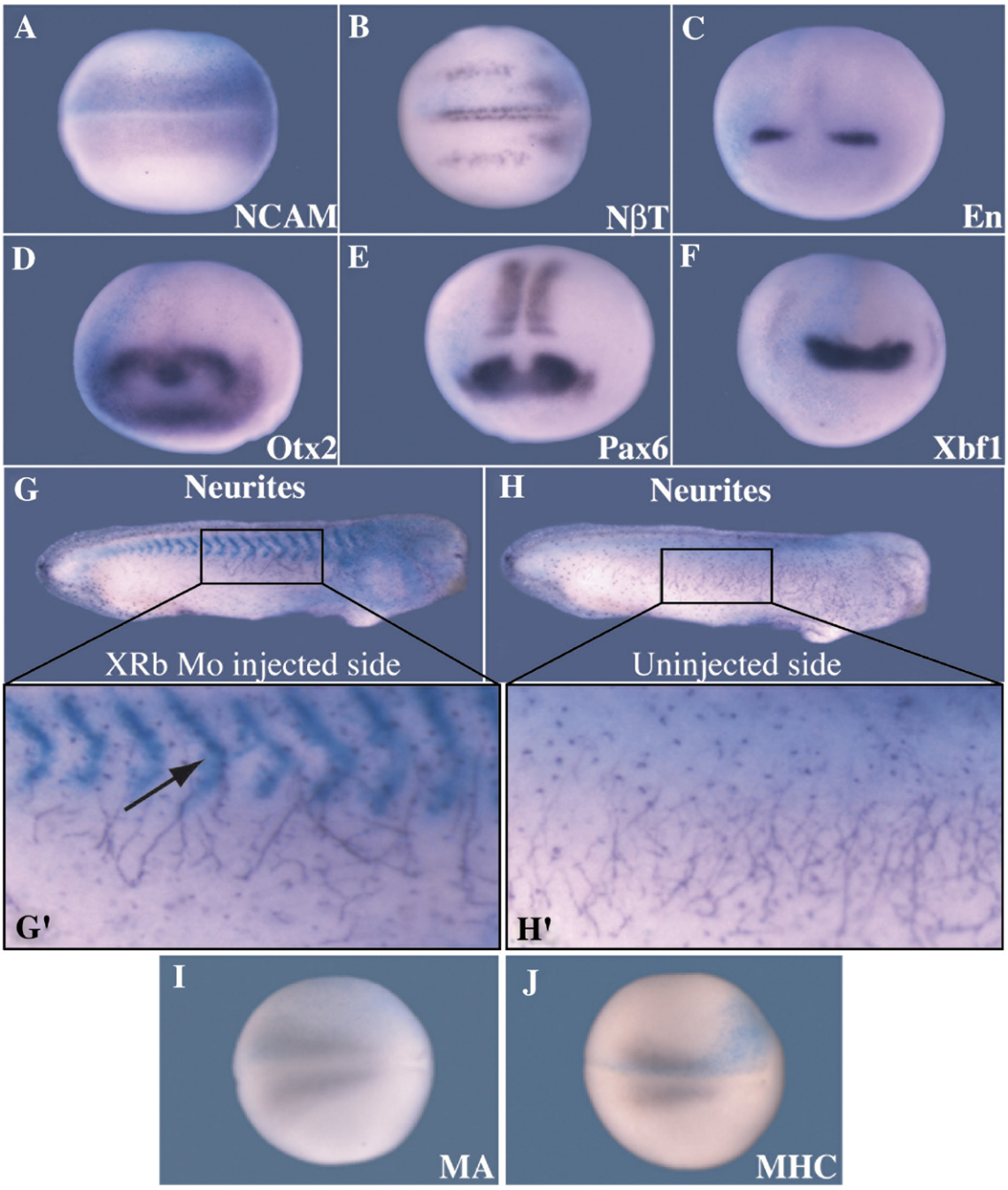

Fig. 3. Depletion of XRb protein does not affect neural or muscle differentiation. Embryos were injected in one blastomere at the two-cell stage with 20 ng XRb Mo with β-galactosidase as a lineage tracer (blue). (A–H) Expression of a range of markers of neural differentiation was examined by in situ hybridisation (stained in purple) at stage 15. NCAM (A); neural β-tubulin (B); Engrailed (C); Otx2 (D); Pax6 (E); Xbf1 (F). Neurite outgrowth was examined by immunostaining for neural β-tubulin at stage 28: XRb Mo-injected side (G) and uninjected side (H) of the same embryo; magnified views of the neurites of the same embryo (G′ and H′). Arrow indicates normal structure of XRb Mo-injected somites (G′). pXRb depletion does not alter the expression patterns of any of these markers. (I, J) Expression of the markers of muscle differentiation muscle actin (I) and myosin heavy chain (J) was visualised by in situ hybridisation (purple) at stage 15: they are not affected by pXRb depletion. Dorsal views; anteriors to the right; injected sides uppermost (A, B, I, J). Anterior views; injected sides to the left (C, D, E and F). Lateral views; anterior to the right (G, G′, H and H′).

with placental insufficiency (de Bruin et al., 2003; Wu et al., 2003). Even if Rb-like functions are essential at these early embryonic stages of *Xenopus* development, it is possible that either enough XRb protein remains after Mo injection, even though it is undetectable by Western blotting, or that other pRb family members such as p107 and p130 can substitute for pRb functions at this time (Dannenberg et al., 2004; Lee et al., 1996; Mulligan et al., 1998; Robanus-Maandag et al., 1998; Ruiz et al., 2004).

A substantial decrease in XRb levels has minimal effects on early *Xenopus* development. However, in mice, increasing absolute amounts of pRb results in a significant decrease in

body weight (Nikitin et al., 2001), indicating cell cycle retardation. In addition, Rb overexpression is sufficient to induce neural differentiation of cultured neuroblastoma cells (Kranenburg et al., 1995). Consequently, we wanted to investigate the effects of *substantial* upregulation in the levels of XRb protein on early *Xenopus* development.

pXRb overexpression

To investigate the effect of XRb overexpression, XRb mRNA was injected into one cell of two-cell stage embryos. A dose-dependent increase in XRb protein level was detected by Western blotting of extracts from XRb mRNA-injected embryos

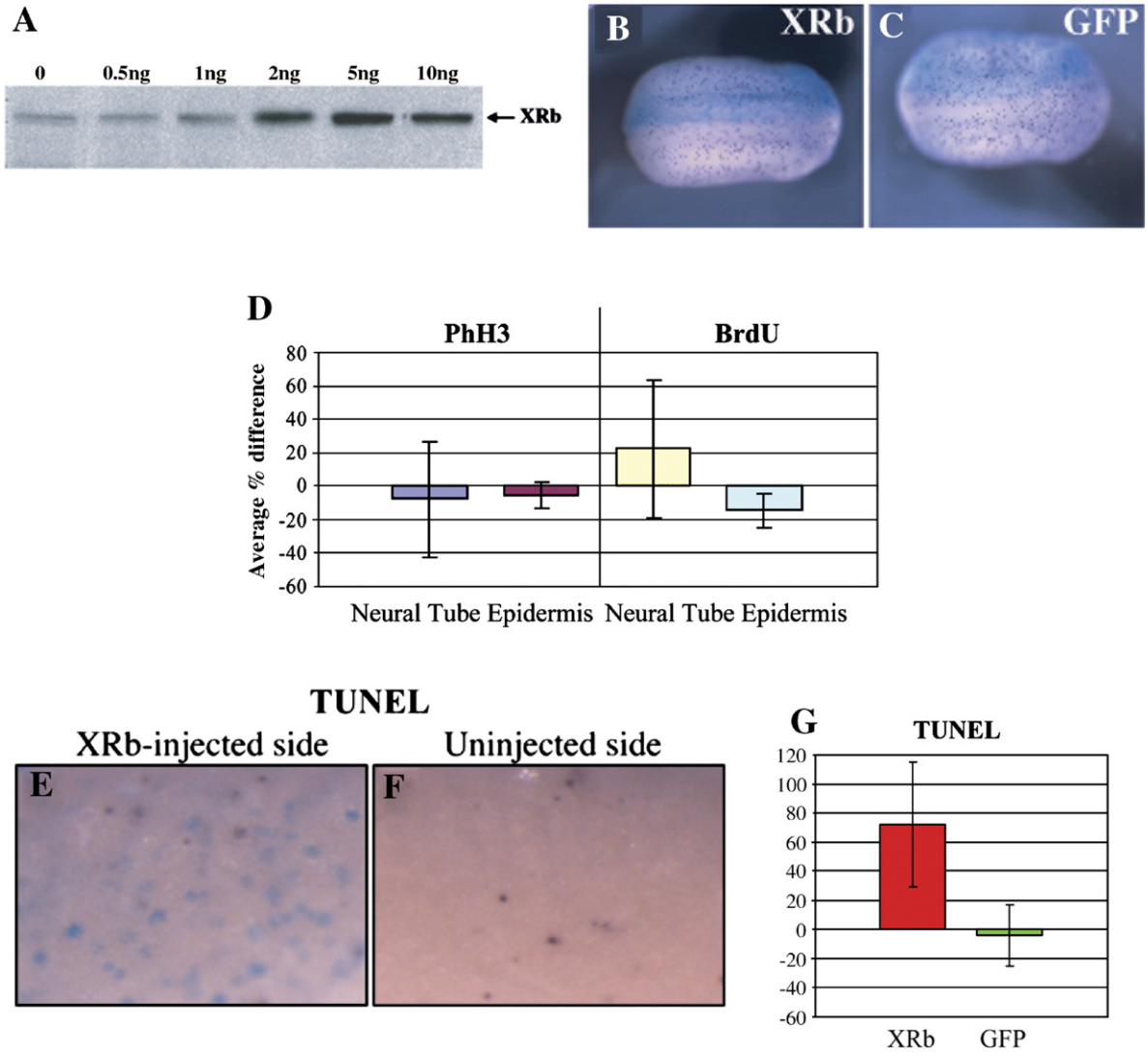

Fig. 4. Overexpression of XRb does not significantly affect the number of cells in mitosis or S phase in the neural tube or epidermis, or the number of apoptotic cells in the epidermis. (A) Embryos were injected in both blastomeres at the two-cell stage with increasing doses of XRb mRNA. Extracts made from stage 19 embryos were Western blotted and probed with an anti-human pRb antibody (XZ160) that cross-reacts with XRb. (B and C) Embryos were injected with 2 ng mRNA encoding XRb or GFP and β-galactosidase (blue) as a marker in one ventral cell at the four-cell stage. At stage 22 epidermal mitotic cells were stained by whole mount immunostaining (purple) with anti-phospho histone H3 antibody. Dorsal views; anteriors to the right; injected sides uppermost. (D) Embryos injected in one dorsal cell at the four-cell stage with 2 ng XRb mRNA and β-galactosidase (blue), with or without BrdU injection at stage 20, were sectioned and immunostained (purple) using anti-phospho histone H3 antibody to visualise mitotic cells or anti-BrdU antibody to visualise cells in S phase. The numbers of positive cells in both injected and uninjected sides of the neural tubes and the epidermis were counted in serial sections: graph shows the average percentage differences between the sides, with error bars showing standard errors ($n=6$ embryos for each marker, average of 39 sections per embryo). XRb overexpression causes no significant difference in the number of mitotic cells on the injected side of the neural tube (average 8% decrease; $p=0.3$) or in the epidermis (average 6% decrease; $p=0.2$), or in the number of cells in S phase in the neural tube (average 22% increase; $p=0.5$) or in the epidermis (average 15% decrease; $p=0.1$). (E and F) Embryos injected with 2 ng XRb mRNA and β-galactosidase (blue) in one cell at the two-cell stage were fixed at stage 22 and apoptotic cells in the epidermis were visualised using whole mount TUNEL staining (purple). XRb overexpression has no effect on the density of apoptotic cells in the epidermis: XRb-injected side (E) and uninjected side (F) of the same embryo. (G) Apoptotic cells were counted in sample areas of the epidermis on both injected and uninjected sides of whole mount TUNEL-stained embryos. Graph shows the average percentage difference between the numbers of apoptotic cells on the two sides of embryos injected with XRb or GRP mRNA, with error bars showing standard errors. XRb overexpression does not make a significant difference to the number of apoptotic cells in the epidermis (average 72% increase; $p=0.09$; $n=8$); GFP injection also causes no difference (average 4% decrease; $p=0.28$; $n=10$).

(Fig. 4A). XRb protein overexpression was achieved which persisted until at least stage 19. Injection of 2 ng of XRb mRNA resulted in significant exogenous expression of XRb protein compared to the endogenous level without affecting embryo viability. Hence, we investigated the effect of pXRb overexpression at this level on cell cycle progression.

Overexpression of XRb protein does not affect cell cycle progression

Firstly we examined the effect of XRb overexpression on mitosis as determined by phospho-histone H3 levels in the neural tube of stage 23 embryos, comparing the uninjected with the injected sides of the neural tube in the same embryos in serial sections. This analysis showed that the number of mitotic cells in the neural tube was not significantly altered by XRb overexpression (Fig. 4D; $p=0.3$; $n=6$, average 38 sections per embryo).

However, XRb is normally highly expressed in the neural tube, so further upregulation may have a limited effect. Instead, we investigated the effect of XRb overexpression in the epidermis where XRb is usually expressed at a low level. Numbers of phospho-histone H3-positive cells were compared on the flank epidermis of the injected and uninjected sides of embryos overexpressing XRb, but again XRb overexpression did not cause a significant difference in the number of cells in mitosis (Figs. 4B and C; $p>0.05$; $n=15$ embryos). We also analysed the number of pH3-expressing cells in the epidermis in sections and saw no significant difference in pH3 expression (Fig. 4D; $p=0.2$; $n=6$ embryos, average 38 sections per embryo). To strengthen this analysis, we also investigated BrdU incorporation on the injected and uninjected sides of embryos unilaterally injected with XRb. Early tailbud stage embryos were sectioned and numbers of BrdU-incorporating cells analysed (Fig. 4D; $p=0.13$; $n=6$, average 39 sections per embryo). Again there was no difference in proliferation in the injected versus the uninjected sides.

To determine whether XRb overexpression resulted in an increase in apoptosis, we also analysed parallel embryos for apoptotic cells using TUNEL staining. Although there was some increase in the average number of apoptotic cells on the XRb-injected side of the embryo compared to the uninjected side, this was not statistically significant when compared with GFP-injected embryos (Figs. 4E–G; pXRb overexpression:

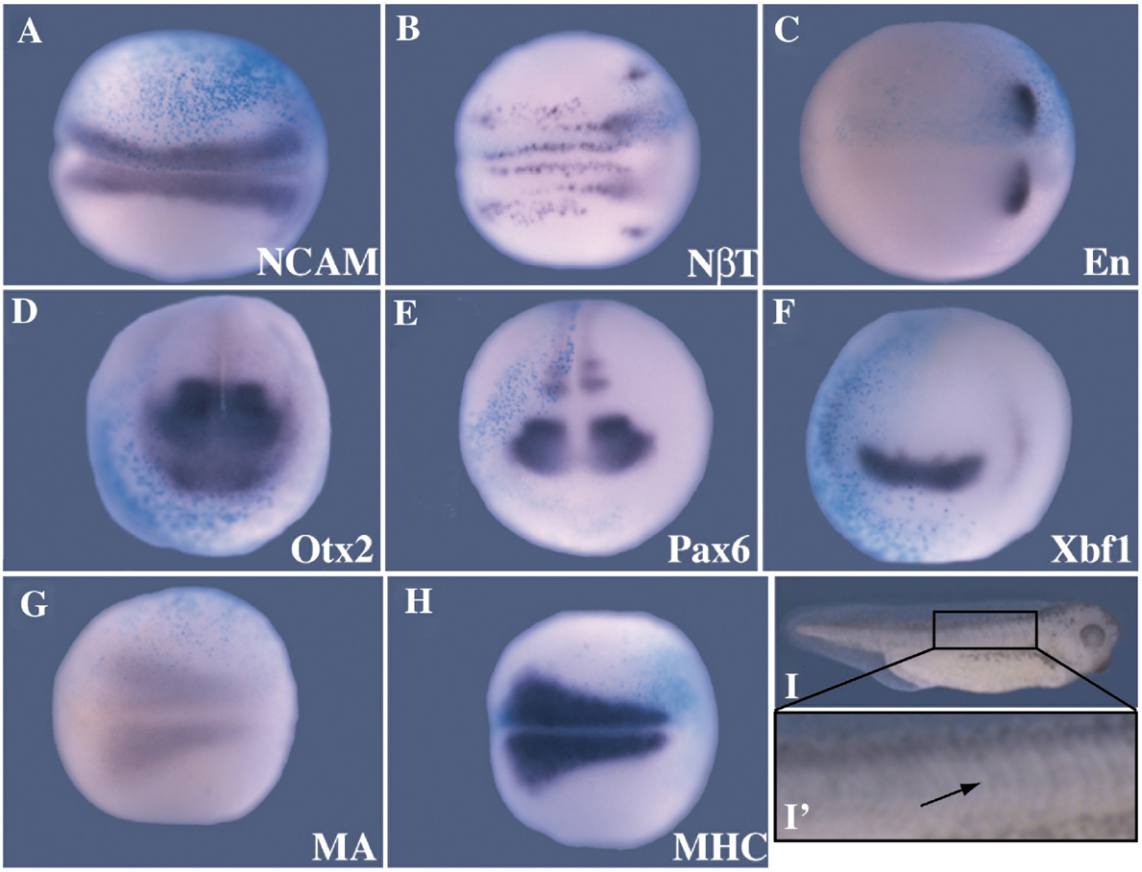

Fig. 5. Overexpression of XRb does not affect neural or muscle differentiation or patterning. Embryos were injected in one blastomere at the two-cell stage with 2 ng XRb mRNA and β-galactosidase as a lineage tracer (blue). (A–H) Expression of a range of markers of neural and muscle differentiation was examined by in situ hybridisation (stained in purple) at stage 15. NCAM (A); neural β-tubulin (B); Engrailed (C); Otx2 (D); Pax6 (E); Xbf1 (F); muscle actin (G); myosin heavy chain (H). The expression patterns of none of these markers were affected by XRb overexpression. Lateral view of an XRb-injected embryo at stage 36 (I) and magnified view of its somites (I′), showing normal somite structure. Arrow indicates normal somite structure (I′). Dorsal views; anterior to the right; injected sides uppermost (A, B, C, G and H). Anterior views; injected sides to the left (D, E and F). Lateral view; anterior to the right (I and I′).

$p=0.09$, $n=8$ embryos; GFP overexpression: $p=0.28$, $n=10$ embryos).

From these analyses, we can conclude that, in early *Xenopus* embryos, overexpression of XRb alone is insufficient to perturb the usual cell cycle regulation of either epidermis or neural tissue.

Overexpression of XRb protein does not affect muscle differentiation

The dual functions of arresting the cell cycle and promoting the transactivation activity of the muscle differentiation factor MEF2 performed by pRb in skeletal myogenesis in mammalian cells are well understood, where the effects of upregulation of pRb levels by transfection were studied (Novitch et al., 1999). In order to perform these functions, Rb is upregulated prior to and during muscle differentiation (Jiang et al., 1997). However, analysis of XRb expression by in situ hybridisation of *Xenopus* embryos does not provide any evidence that XRb is upregulated in the myotome (Fig. 1).

If pXRb is capable of promoting differentiation, its overexpression might be expected to exert an effect on either cell cycle exit and/or cell type-specific gene expression in tissues in which it is endogenously expressed at a low level, such as the myotome. The effect of pXRb overexpression on muscle differentiation was therefore examined by injecting embryos in one blastomere at the two-cell stage with XRb mRNA and β-gal mRNA as a lineage marker and performing whole mount in situ hybridisation for the markers of muscle specification and differentiation, muscle actin and myosin heavy chain, at stage 15. Both of these markers were expressed normally in both the pXRb-injected sides and the uninjected sides of the embryos (Figs. 5G, H; $n=40$ per marker), and embryos developed normally to later stages. This indicates that pXRb overexpression does not perturb muscle differentiation in *Xenopus* embryos. Moreover, older embryos overexpressing pXRb are morphologically normal, and fully formed somitic blocks are clearly visible (Figs. 5I, I′).

Overexpression of XRb protein does not affect neural differentiation

The role of XRb protein in neural differentiation was also examined. The expression patterns of the markers of terminal neural differentiation, neural β-tubulin (NβT) and NCAM, and of the regional neural markers Engrailed, Otx2, Pax6 and Xbf1 were visualised by whole mount in situ hybridisation. They were not affected by pXRb protein overexpression (Figs. 5A–F, $n=40$ per marker). These results show that pXRb overexpression perturbs neither neural specification nor differentiation.

Phosphorylation of pXRb during early embryogenesis

Surprisingly, and in contrast to results found in mammalian embryos (Bignon et al., 1993; Nikitin et al., 2001), overexpression of pXRb had no discernable effect on early *Xenopus* embryogenesis. To ascertain whether exogenous XRb protein was active, its phosphorylation status was investigated. pRb is primarily active in binding E2Fs and recruiting chromatin repressors when it is hypophosphorylated; phosphorylation by cyclin/CDK complexes inactivates it and prevents its interaction with E2Fs, allowing E2F-mediated transactivation of targets to promote cell cycle progression and DNA replication (Sherr, 2000).

In order to determine the phosphorylation status of exogenous pXRb, embryo extracts from different stages were treated with lambda phosphatase. Western blots of treated and untreated XRb-injected embryo extracts showed that XRb protein first becomes detectable around stage 8. Beyond this, at all early stages tested, lambda phosphatase treatment caused an increase in electrophoretic mobility indicative of decreased molecular weight of endogenous XRb (Fig. 6A). However, pXRb extracted from considerably older metamorphosing stage 50 embryos consisted of both a slower and faster migrating species (Fig. 6B). The fastest migrating pXRb from stage 50 embryos co-migrates with pXRb from stage 22 embryos that has been treated with lambda phosphatase, demonstrating that at this late stage of development pXRb exists in a hyper- and a hypo-phosphorylated form. Moreover, exogenous pXRb also migrates faster after lambda phosphatase incubation (Fig. 6C: compare lanes 3 and 4). This result

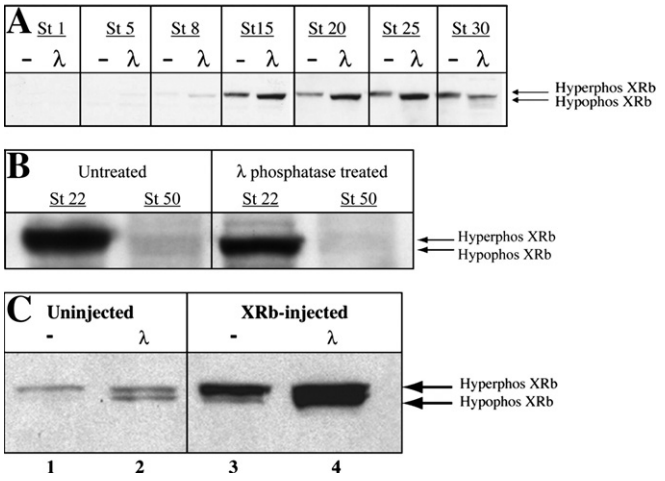

Fig. 6. XRb is predominantly hyperphosphorylated during *Xenopus* embryogenesis. (A) Extracts made from embryos at a range of stages between 1 and 30 were either untreated (−) or treated with lambda phosphatase (λ) and immunoblotted with anti-Rb antibody (XZ160). Endogenous Rb protein is detected at all stages between 8 and 30 in its hyperphosphorylated form (upper bands). (B) Extracts made from embryos at stage 22 (30 μg total protein) or stage 50 (50 μg total protein) were either untreated (−) or treated with lambda phosphatase (λ) and immunoblotted with anti-Rb antibody (XZ160). pXRb was predominantly hyperphosphorylated at stage 22 but exists in hyper- and hypophosphorylated forms at stage 50. (C) Extracts made from uninjected or XRb mRNA (2 ng)-injected embryos at stage 22 were either untreated (lanes 1 and 3) or treated with lambda phosphatase (lanes 2 and 4) and immunoblotted with anti-Rb antibody (XZ160). Both endogenous pXRb, seen in uninjected extracts (lanes 1 and 2), and exogenous pXRb, seen in extracts from XRb-injected embryos (lanes 3 and 4), are predominantly in the hyperphosphorylated form (upper bands) at stage 22. Hyperphosphorylated XRb (Hyperphos XRb); hypophosphorylated XRb (Hypophos XRb); stage (St).

demonstrates that pXRb is hyperphosphorylated and therefore in its inactive form in vivo at these stages of development. This inactivity explains the inactivity of pXRb translated from injected mRNA and also the apparent dispensability of pXRb during early *Xenopus* embryogenesis.

Discussion

Numerous papers over almost 20 years have investigated the role of pRb in cell cycle progression. While Rb is expressed in all embryonic and adult tissues examined in the mouse (Bernards et al., 1989), the level of Rb expression in mouse tissues varies significantly according to cell type and differentiation status (Jiang et al., 1997; Szekely et al., 1992). More recently, it has become apparent that, in addition to controlling the cell cycle (Weinberg, 1995), pRb has complementary roles in the regulation of differentiation, apoptosis (Morgenbesser et al., 1994; Pan and Griep, 1995), senescence (Dannenberg et al., 2000; Stein et al., 1990) and maintenance of stem cell identity (Wildwater et al., 2005). Thus, its expression pattern during development may correlate with the roles pRb plays in differentiation, firstly to implement cell cycle arrest (Juan et al., 1998) and secondly to promote cell type-specific gene expression (Korenjak and Brehm, 2005; Sellers et al., 1998).

The molecular details of these roles have been primarily established in cultured cell systems. However, a more complex situation has been revealed by analysis of pRb function in vivo by generation of Rb null mice. Classic Rb knockout mice, in which pRb is missing from both embryonic and extra-embryonic tissues, show extensive defects particularly in the central nervous and haematopoietic systems, with resultant widespread apoptosis, leading to lethality around E14.5 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The hypothesis was proposed that pRb was essential for correct cell cycle exit and differentiation in these tissues. However, it was subsequently discovered that pRb is essential for formation of the placenta. Mice in which Rb has only been knocked out in the embryonic tissue but where the placenta is Rb+/+ show essentially normal differentiation of the central nervous system and haematopoiesis. However, these embryos still die perinatally, exhibiting severe defects in skeletal muscle formation (de Bruin et al., 2003; Wu et al., 2003). Subsequent studies have demonstrated both cell autonomous and cell non-autonomous functions of pRb during early mouse development (Maandag et al., 1994; Williams et al., 1994), but earlier results must now be reinterpreted in the light of the demonstrated placental compromise in the Rb null mice.

We were interested in investigating the function of pRb in early development at initial stages of tissue differentiation, and to this end have investigated the effects of altered pRb levels on cell cycle regulation and differentiation of early *Xenopus* embryos, which develop outside the mother without requiring placental function. Initially, we determined the distribution of XRb transcripts during early embryogenesis by in situ hybridisation (Fig. 1). XRb is found at very low levels early in development. As development progresses, XRb transcripts accumulate specifically in the central nervous system and the neural crest, areas where proliferation is high. In contrast, XRb is undetectable in the myotome. Also, XRb cannot be detected by in situ hybridisation in the epidermis, but can be seen at low levels in differentiated animal caps by RT-PCR (data not shown).

Very early *Xenopus* cell cycles, prior to the mid-blastula transition (MBT), consist of alternating S and M phases. However, after MBT the cell cycle lengthens to incorporate G1 and G2 phases, and the cell cycle is assumed to more closely resemble that of mammalian cultured cells (Newport and Kirschner, 1982). Firstly, we investigated whether depleting pXRb could alter cell cycling post-MBT by microinjection of an antisense morpholino oligonucleotide designed to prevent XRb mRNA translation. This was achieved very efficiently, with pXRb undetectable by Western blot at least until tailbud stage 23 (Fig. 2A). Surprisingly, pXRb depletion had no effect on cell cycling in either the neural tube (Figs. 2B, D), where pXRb is highly expressed, or in the epidermis (Fig. 2C), where it is found at lower levels. Initial analysis was performed by counting cells in mitosis that expressed phospho-histone H3. However, *Drosophila* embryos that have been depleted of the related protein RBF undergo ectopic S phases, as measured by BrdU incorporation, but these cells fail to proceed to mitosis, and this is accompanied by widespread apoptosis (Du and Dyson, 1999). Moreover, pRb itself has been shown to be a direct regulator of apoptosis (Morgenbesser et al., 1994; Pan and Griep, 1995). Therefore, we determined the number of cells incorporating BrdU in the morpholino-injected side of the neural tube compared to the uninjected side and again, saw no difference (Figs. 2Ca–f). Moreover, the number of apoptotic cells, as visualised by TUNEL staining, was also unaffected (Figs. 2E, F). Thus, loss of pXRb has no detectable effect on cell cycling or apoptosis in the early embryo.

The absolute amount of Rb protein has been shown to be important in mammalian systems, with simple overexpression resulting in a number of phenotypes. Overexpression of pRb in mouse embryos results in a decrease in body weight of between 10 and 30% at gestational day 12.5, when compared with their wild-type littermates (Nikitin et al., 2001). This weight reduction is a dose-dependent effect of Rb overexpression: the degree of dwarfism correlates roughly with the copy number of the Rb transgene used to overexpress Rb and the level of Rb protein (Bignon et al., 1993). Overexpression of Rb in normal cultured cells also results in growth arrest (Fung et al., 1993). Moreover, the level of pRb affects the differentiation status of cultured neuroblastoma cells: Rb overexpression is sufficient to induce their neural differentiation (Kranenburg et al., 1995). These results show that growth regulation in early embryogenesis and the differentiation of some cell types are sensitive to the absolute amount of pRb available. However, in *Xenopus* early embryos, we show that pXRb overexpression has no effect on cell cycling and little effect on apoptosis.

We then went on to investigate whether perturbing levels of pXRb alter expression of either regional neural markers or markers of neural differentiation, including the terminal differentiation marker neural β-tubulin. Again, neither depletion of XRb protein using morpholinos nor overexpression by mRNA microinjection resulted in altered expression of either early markers of regional neural identity such as Engrailed (En) or Otx2 or markers of early or late neural differentiation (NCAM and neural β-tubulin respectively) (Figs. 3A–F and 5A–F). Loss of pRb in primary mouse cell cultures has been shown to affect neurite outgrowth (Lee et al., 1994). We assayed this in vivo in *Xenopus* embryos after XRb Mo injection and again, we saw no alteration in the pattern of neurites on the injected versus the uninjected side (Figs. 3G, H).

In mouse embryos, pRb is thought to be particularly important for skeletal muscle differentiation (Gu et al., 1993). It has been suggested that pRb and MyoD co-operate to activate the transcriptional activity of MEF2, as well as to effect permanent cell cycle withdrawal in myoctyes by upregulating CDK inhibitor expression (Novitch et al., 1999). Somitic muscle differentiation in early *Xenopus* embryos is thought to occur by analogous pathways, so we investigated whether differentiation of the myotome, which occurs early in *Xenopus* development, is affected by pXRb levels. Neither depletion nor overexpression of pXRb affected the expression of the early muscle marker muscle actin or the late marker heavy chain myosin (Figs. 3I, J and 5G, H). Moreover, embryos that were allowed to develop to later stages appeared to develop normal somitic muscle (see β-gal expression in somites formed after XRb Mo injection, Fig. 3G). Therefore, pXRb does not regulate somitic muscle differentiation at these stages.

Considering the central role that has been described for pRb in the regulation of the cell cycle and differentiation in other species, what are we to make of these results? Rb is a member of a family of "pocket proteins" consisting of Rb, p107 and p130 (for a review, see Lipinski and Jacks, 1999). In mice, p107 and p130 are thought to have partially overlapping functions and moreover, when Rb is knocked out, its loss can be partially compensated for by upregulation of p107 (Schneider et al., 1994). *Xenopus* homologues of p107 and p130 have not been published, but preliminary investigation of the genome of the closely related species, *Xenopus tropicalis* shows that homologues of both proteins are present (data not shown). Therefore, it is likely that these may compensate for the loss of XRb.

Additionally, while our morpholino directed against XRb appears highly efficient by Western blot analysis, it is possible that low levels of pXRb remain. However, it is clear that in the mouse model, absolute levels of pRb are important. Rb null mice die at E14.5 (Jacks et al., 1992), but the partial rescue of Rb null mice by Rb expression from a minigene at approximately 50% of the wild-type level (Zacksenhaus et al., 1996) shows that a low level of Rb protein is sufficient for the performance of some, but not all, of pRb's functions. These mice survive beyond gestational day 14.5, but die at birth with specific skeletal muscle defects resulting from reduced myoblast survival and impaired cell cycle withdrawal (Zacksenhaus et al., 1996), indicating that a higher level of pRb is required for the fulfillment of its role in muscle differentiation than other functions. Moreover, Rb heterozygous mice expressing Rb at 60% of the wild-type level are 5% larger than their littermates, while Rb null mice are 15% larger than wild-type at gestational day 12.5 (Nikitin et al., 2001), demonstrating the importance of absolute levels of pRb.

We propose that XRb (and potentially other pocket proteins) are not required for cell cycle regulation or differentiation in *Xenopus* at these early embryonic stages. This hypothesis is supported by three lines of evidence. Firstly, we have demonstrated previously that *Xenopus* E2F is present in early embryos in a form that binds to DNA but does not show significant complexing to pXRb until stage 17, by gel retardation assay, and even then pXRb only binds to a very small proportion of E2F. Moreover, only a single gel-retarded form of E2F containing pXRb is evident in early embryos, while E2F activity from cultured XTC cells, derived from much older metamorphosing *Xenopus* froglets, is composed of free E2F and two slower migrating forms (Philpott and Friend, 1994). Thus, in early embryos, E2F complexes are more simple than those found in cells derived from older frogs.

Secondly, active pRb, which interacts with E2F and other factors to regulate passage through G1 phase, cell cycle exit and differentiation, is known to be hypophosphorylated (Weinberg, 1995). In contrast, we demonstrate here that the overwhelming majority of pXRb at early embryonic stages is hyperphosphorylated (Fig. 6). This is not only true for endogenous pXRb but also for overexpressed pXRb. Therefore, at least until stage 30, pXRb is primarily maintained in an inactive form, again indicating that it is not essential for cell cycle progression or differentiation at these stages of development, and this would explain why upregulation or downregulation of pXRb levels has no developmental effect early on.

Thirdly, in mouse embryos it has been clearly shown that there is a cell-autonomous requirement for pRb in differentiation of muscle (Gu et al., 1993). Interestingly, not only is pRb required for stable cell cycle exit in this tissue (Novitch et al., 1996), but it must also co-operate with MyoD to bring about the full programme of muscle differentiation, possibly by promoting activity of the transcription factor MEF2 (Novitch et al., 1999). Perhaps surprisingly, considering the wealth of data demonstrating a role for pRb in muscle differentiation, XRb transcripts are not detected in the developing embryonic myotome. Moreover, neither early nor late markers of muscle differentiation are perturbed by either overexpression or loss of pXRb (Figs. 3I, J and 5G, H). So clearly, at these developmental stages, muscle differentiation in *Xenopus* is not regulated by pXRb levels.

Analyses of Rb null mice have shown that significant defects become apparent only relatively late in embryonic gestation (for a review see Lipinski and Jacks, 1999). Moreover, many of the most dramatic phenotypes that were described originally resulted from the cell non-autonomous effect of hypoxia induced by placental defects (Wu et al., 2003). Our data indicate that pXRb is inactive at early embryonic stages in *Xenopus*. Inactivity of pRb during early embryogenesis has also been described in mouse embryos:

the first pRb to be detected around day 10.5 of gestation is mainly phosphorylated, with the hypophosphorylated form only appearing in significant amounts later on (Paggi et al., 1996). In mouse embryonic stem cells, pRb is also found to be mainly hyperphosphorylated; this has been correlated with their high rate of proliferation (Savatier et al., 1994). The maintenance of pRb in its inactive state during early embryogenesis may therefore facilitate the high level of cell proliferation required for the rapid growth of young embryos. However, as cells exit the cell cycle during their progression along the various differentiation pathways, pRb activity might be expected to be required to regulate the commitment of cells to continued cycling. The sustained inactivity of pRb during differentiation stages of early embryos may indicate that other Rb-related proteins are active during early embryogenesis to promote cell cycle exit and differentiation, although alternative regulatory pathways are also possible. However, it is clear that embryonic cell cycles post-mid-blastula transition, while adopting the usual G1, S, G2, M progression, may control the transition between stages somewhat differently to somatic cells.

Thus, Rb clearly plays a central role in cell cycle regulation and differentiation of a wide variety of tissues. However, these roles may vary greatly depending on embryonic stage, activity of other pocket proteins and mode of development, e.g., placental versus non-placental. Our surprising results show that we must study pRb in the widest possible context if we are to fully understand its importance in cell cycle regulation and differentiation in vivo. Moreover, we have much yet to discover about the unusual ways in which the cell cycle and differentiation are regulated during embryonic development, and must look to in vivo models to address these questions.

Experimental procedures

Embryos and injections

X. laevis embryos obtained by hormone-induced laying were fertilised in vitro, dejellied in 2% cysteine, pH 7.8–8.0, washed and incubated in 0.1× MBS. Embryos were staged according to Nieuwkoop and Faber (1984), fixed in MEMFA and stained for beta-galactosidase (β-gal: 500 pg–1 ng injected per embryo as a lineage tracer) as described (Sive et al., 2000). Embryos were depigmented in 10% H_2O_2 with 5% formamide and 0.1% SSC in PBS over bright white light.

The full coding sequence of *Xenopus* Rb (Destree et al., 1992) was subcloned into pCS2+ and transcribed using the SP6 Message Machine kit (Ambion). The sequence of the anti-XRb morpholino was: 5′-GTTTTCTAGGGCTCTTTGGAGGCAT-3′; and the sequence of the control morpholino (targeted at a mutant sequence of human β-globin pre-mRNA) was: 5′-CCTCTTACCTCAGTTACAATTTATA-3′ (Gene Tools). Typically 2 ng of mRNA or 20 ng morpholino was injected into embryos at the two-cell stage in 0.2× MBS supplemented with 4% Ficoll.

Western blotting

Protein extracts were prepared from whole embryos as described (Philpott and Friend, 1994). Total protein was separated by SDS-PAGE, Western blotted to nitrocellulose by standard methods and probed for XRb using anti-Rb antibody XZ160 (Hu et al., 1991) and an HRP-conjugated anti-mouse antibody (Amersham), or with an anti-β-actin antibody (Abcam) and an HRP-conjugated rabbit antibody (Jackson Immunoresearch). Antibody binding was detected using SuperSignal chemiluminescence detection (Pierce).

In situ hybridisation, BrdU detection, TUNEL and antibody staining

Whole mount in situ hybridisation was performed as described (Shimamura et al., 1994), using the alkaline phosphatase substrate BM purple (Roche). Linearised plasmids encoding XRb (ClaI/T7), muscle actin (HindIII/T7), myosin heavy chain (NcoI/SP6), NCAM (ClaI/SP6), neural β-tubulin (BamHI/T3), Engrailed (XbaI/T3), Otx2 (NotI/T7), Pax6 (NotI/T7) and Xbf1 (XhoI/SP6) were used to generate digoxigenin-labelled (Roche) antisense RNA probes using the restriction enzymes and RNA polymerases indicated.

BrdU incorporation and detection were performed as described (Hardcastle and Papalopulu, 2000). Whole mount antibody staining was performed as described (Sive et al., 2000), using anti-phospho-histone H3 antibody (Upstate) at 1:500 or anti-neural β-tubulin antibody (JDR 3BS) at 1:350, detected with alkaline phosphatase-conjugated secondary antibodies (Jackson Immunoresearch and Sigma) using NBT/BCIP (Roche) as substrates. Whole mount TUNEL staining was performed as described (Hensey and Gautier, 1998).

Embryo sectioning and immunohistochemistry

Embryos were fixed in 4% paraformaldehyde, soaked in 30% sucrose solution and embedded in OCT medium (Sakura Tissue-Tek) before being sectioned by cryostat at 10 μm. Mitotic cells in sectioned embryos were detected using anti-phospho-histone H3 antibody (Upstate; Saka and Smith, 2001), diluted 1:300 and an AlexaFluor-conjugated anti-rabbit secondary antibody (Molecular Probes). BrdU incorporation was detected using anti-BrdU antibody (Roche), diluted 1:100 with an AlexaFluor-conjugated anti-mouse secondary antibody (Molecular Probes). Nuclei were visualised using Hoechst staining.

Statistical analysis

Statistical analysis was performed using paired *t*-tests to analyse cell numbers, comparing the sum of cells counted on the injected and uninjected sides of all sections of each embryo. We calculated the percentage difference between the two sides for each embryo, and then calculated the average for the six embryos of each group, as depicted on the graphs, with error bars showing the standard errors of the means. For the pH3 analysis, on average 6 pH3-positive cells per embryo were seen in the neural tube and 271 pH3-positive cells were seen in the epidermis. For BrdU analysis, on average 25 positive cells were seen in the neural tube per embryo and 1243 positive cells were seen in the epidermis.

λ-phosphatase treatment of embryo extracts

Embryo extracts were incubated with 100 units λ-phosphatase (NEB) in λ-PPase buffer (NEB) with 2 mM $MnCl_2$ for 3 h at 30°C and then electrophoresed by SDS-PAGE prior to Western blotting.

Acknowledgments

We are grateful to Nick Dyson and Ed Harlow for generously supplying anti-Rb antibodies, Olivier Destree and Stephen Friend for XRb, Laurent Richard-Parpaillon for samples, Ian Horan for technical help and to Shinichi Ohnuma and Phil Jones for their helpful discussions. R.A.C. was supported by a Cancer Research UK studentship. A.P. was supported by BBSRC grant BB/C004108/1.

References

Balak, K., Jacobson, M., Sunshine, J., Rutishauser, U., 1987. Neural cell adhesion molecule expression in *Xenopus* embryos. Dev. Biol. 119, 540–550.

Batsche, E., Moschopoulos, P., Desroches, J., Bilodeau, S., Drouin, J., 2005. Retinoblastoma and the related pocket protein p107 act as coactivators of NeuroD1 to enhance gene transcription. J. Biol. Chem. 280, 16088–16095.

Bernards, R., Shackleford, G.M., Gerber, M.R., Horowitz, J.M., Friend, S.H., Schartl, M., Bogenmann, E., Rapaport, J.M., McGee, T., Dryja, T.P., et al., 1989. Structure and expression of the murine retinoblastoma gene and characterization of its encoded protein. Proc. Natl. Acad Sci. U. S. A. 86, 6474–6478.

Bignon, Y.J., Chen, Y., Chang, C.Y., Riley, D.J., Windle, J.J., Mellon, P.L., Lee, W.H., 1993. Expression of a retinoblastoma transgene results in dwarf mice. Genes Dev. 7, 1654–1662.

Bourguignon, C., Li, J., Papalopulu, N., 1998. XBF-1, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in *Xenopus* competent ectoderm. Development 125, 4889–4900.

Chen, P.L., Riley, D.J., Chen, Y., Lee, W.H., 1996. Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev. 10, 2794–2804.

Clarke, A.R., Maandag, E.R., van Roon, M., van der Lugt, N.M., van der Valk, M., Hooper, M.L., Berns, A., te Riele, H., 1992. Requirement for a functional Rb-1 gene in murine development. Nature 359, 328–330.

Dannenberg, J.H., van Rossum, A., Schuijff, L., te Riele, H., 2000. Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev. 14, 3051–3064.

Dannenberg, J.H., Schuijff, L., Dekker, M., van der Valk, M., te Riele, H., 2004. Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev. 18, 2952–2962.

de Bruin, A., Wu, L., Saavedra, H.I., Wilson, P., Yang, Y., Rosol, T.J., Weinstein, M., Robinson, M.L., Leone, G., 2003. Rb function in extraembryonic lineages suppresses apoptosis in the CNS of Rb-deficient mice. Proc. Natl. Acad Sci. U. S. A. 100, 6546–6551.

Destree, O.H., Lam, K.T., Peterson-Maduro, L.J., Eizema, K., Diller, L., Gryka, M.A., Frebourg, T., Shibuya, E., Friend, S.H., 1992. Structure and expression of the *Xenopus* retinoblastoma gene. Dev. Biol. 153, 141–149.

Du, W., Dyson, N., 1999. The role of RBF in the introduction of G1 regulation during *Drosophila* embryogenesis. EMBO J. 18, 916–925.

Dyson, N., 1998. The regulation of E2F by pRB-family proteins. Genes Dev. 12, 2245–2262.

Eizema, K., Koster, J.G., Stegeman, B.I., Baarends, W.M., Lanser, P.H., Destree, O.H., 1994. Comparative analysis of Engrailed-1 and Wnt-1 expression in the developing central nervous system of *Xenopus laevis*. Int. J. Dev. Biol. 38, 623–632.

Flemington, E.K., Speck, S.H., Kaelin Jr., W.G., 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc. Natl. Acad Sci. U. S. A. 90, 6914–6918.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M., Dryja, T.P., 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323, 643–646.

Fung, Y.K., T'Ang, A., Murphree, A.L., Zhang, F.H., Qiu, W.R., Wang, S.W., Shi, X.H., Lee, L., Driscoll, B., Wu, K.J., 1993. The Rb gene suppresses the growth of normal cells. Oncogene 8, 2659–2672.

Gu, W., Schneider, J.W., Condorelli, G., Kaushal, S., Mahdavi, V., Nadal-Ginard, B., 1993. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell 72, 309–324.

Hardcastle, Z., Papalopulu, N., 2000. Distinct effects of XBF-1 in regulating the cell cycle inhibitor p27(XIC1) and imparting a neural fate. Development 127, 1303–1314.

Hartenstein, V., 1989. Early neurogenesis in *Xenopus*: the spatio-temporal pattern of proliferation and cell lineages in the embryonic spinal cord. Neuron 3, 399–411.

Heasman, J., 2002. Morpholino oligos: making sense of antisense? Dev. Biol. 243, 209–214.

Hensey, C., Gautier, J., 1998. Programmed cell death during *Xenopus* development: a spatio-temporal analysis. Dev. Biol. 203, 36–48.

Hirsch, N., Harris, W.A., 1997. *Xenopus* Pax-6 and retinal development. J. Neurobiol. 32, 45–61.

Hu, Q.J., Bautista, C., Edwards, G.M., Defeo-Jones, D., Jones, R.E., Harlow, E., 1991. Antibodies specific for the human retinoblastoma protein identify a family of related polypeptides. Mol. Cell. Biol. 11, 5792–5799.

Jacks, T., Fazeli, A., Schmitt, E.M., Bronson, R.T., Goodell, M.A., Weinberg, R.A., 1992. Effects of an Rb mutation in the mouse. Nature 359, 295–300.

Jiang, Z., Zacksenhaus, E., Gallie, B.L., Phillips, R.A., 1997. The retinoblastoma gene family is differentially expressed during embryogenesis. Oncogene 14, 1789–1797.

Jiang, Z., Guo, Z., Saad, F.A., Ellis, J., Zacksenhaus, E., 2001. Retinoblastoma gene promoter directs transgene expression exclusively to the nervous system. J. Biol. Chem. 276, 593–600.

Juan, G., Li, X., Darzynkiewicz, Z., 1998. Phosphorylation of retinoblastoma protein assayed in individual HL-60 cells during their proliferation and differentiation. Exp. Cell Res. 244, 83–92.

Korenjak, M., Brehm, A., 2005. E2F-Rb complexes regulating transcription of genes important for differentiation and development. Curr. Opin. Genet. Dev. 15, 520–577.

Kranenburg, O., Scharnhorst, V., Van der Eb, A.J., Zantema, A., 1995. Inhibition of cyclin-dependent kinase activity triggers neuronal differentiation of mouse neuroblastoma cells. J. Cell Biol. 131, 227–234.

Lee, E.Y., Chang, C.Y., Hu, N., Wang, Y.C., Lai, C.C., Herrup, K., Lee, W.H., Bradley, A., 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 359, 288–294.

Lee, E.Y., Hu, N., Yuan, S.S., Cox, L.A., Bradley, A., Lee, W.H., Herrup, K., 1994. Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. Genes Dev. 8, 2008–2021.

Lee, M.H., Williams, B.O., Mulligan, G., Mukai, S., Bronson, R.T., Dyson, N., Harlow, E., Jacks, T., 1996. Targeted disruption of p107: functional overlap between p107 and Rb. Genes Dev. 10, 1621–1632.

Lipinski, M.M., Jacks, T., 1999. The retinoblastoma gene family in differentiation and development. Oncogene 18, 7873–7882.

Maandag, E.C., van der Valk, M., Vlaar, M., Feltkamp, C., O'Brien, J., van Roon, M., van der Lugt, N., Berns, A., te Riele, H., 1994. Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. EMBO J. 13, 4260–4268.

Morgenbesser, S.D., Williams, B.O., Jacks, T., DePinho, R.A., 1994. p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature 371, 72–74.

Muller, H., Bracken, A.P., Vernell, R., Moroni, M.C., Christians, F., Grassilli, E., Prosperini, E., Vigo, E., Oliner, J.D., Helin, K., 2001. E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev. 15, 267–285.

Mulligan, G.J., Wong, J., Jacks, T., 1998. p130 is dispensable in peripheral T lymphocytes: evidence for functional compensation by p107 and pRB. Mol. Cell. Biol. 18, 206–220.

Nead, M.A., Baglia, L.A., Antinore, M.J., Ludlow, J.W., McCance, D.J., 1998. Rb binds c-Jun and activates transcription. EMBO J. 17, 2342–2352.

Newport, J., Kirschner, M., 1982. A major developmental transition in early *Xenopus* embryos: I. characterization and timing of cellular changes at the midblastula stage. Cell 30, 675–686.

Nieuwkoop, P.D., Faber, J., 1984. Normal table of *Xenopus laevis*. Garland Publishing, New York.

Nikitin, A., Shan, B., Flesken-Nikitin, A., Chang, K.H., Lee, W.H., 2001. The retinoblastoma gene regulates somatic growth during mouse development. Cancer Res. 61, 3110–3118.

Novitch, B.G., Mulligan, G.J., Jacks, T., Lassar, A.B., 1996. Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. J. Cell Biol. 135, 441–456.

Novitch, B.G., Spicer, D.B., Kim, P.S., Cheung, W.L., Lassar, A.B., 1999. pRb is required for MEF2-dependent gene expression as well as cell-cycle arrest during skeletal muscle differentiation. Curr. Biol. 9, 449–459.

Paggi, M.G., Baldi, A., Bonetto, F., Giordano, A., 1996. Retinoblastoma protein family in cell cycle and cancer: a review. J. Cell. Biochem. 62, 418–430.

Pan, H., Griep, A.E., 1995. Temporally distinct patterns of p53-dependent and p53-independent apoptosis during mouse lens development. Genes Dev. 9, 2157–2169.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G., Boncinelli, E., 1995. The *Xenopus* homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions. Development 121, 707–720.

Philpott, A., Friend, S.H., 1994. E2F and its developmental regulation in *Xenopus laevis*. Mol. Cell. Biol. 14, 5000–5009.

Richter, K., Grunz, H., Dawid, I.B., 1988. Gene expression in the embryonic nervous system of *Xenopus laevis*. Proc. Natl. Acad Sci. U. S. A. 85, 8086–8090.

Robanus-Maandag, E., Dekker, M., van der Valk, M., Carrozza, M.L., Jeanny, J. C., Dannenberg, J.H., Berns, A., te Riele, H., 1998. p107 is a suppressor of retinoblastoma development in pRb-deficient mice. Genes Dev. 12, 1599–1609.

Ruiz, S., Santos, M., Segrelles, C., Leis, H., Jorcano, J.L., Berns, A., Paramio, J.M., Vooijs, M., 2004. Unique and overlapping functions of pRb and p107 in the control of proliferation and differentiation in epidermis. Development 131, 2737–2748.

Saka, Y., Smith, J.C., 2001. Spatial and temporal patterns of cell division during early *Xenopus* embryogenesis. Dev. Biol. 229, 307–318.

Savatier, P., Huang, S., Szekely, L., Wiman, K.G., Samarut, J., 1994. Contrasting patterns of retinoblastoma protein expression in mouse embryonic stem cells and embryonic fibroblasts. Oncogene 9, 809–818.

Schneider, J.W., Gu, W., Zhu, L., Mahdavi, V., Nadal-Ginard, B., 1994. Reversal of terminal differentiation mediated by p107 in Rb−/− muscle cells. Science 264, 1467–1471.

Sellers, W., Novitch, B.G., Miyake, S., Heith, A., Otterson, G.A., Kaye, F. J., Lassar, A.B., Kaelin Jr., W.G., 1998. Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth. Genes Dev. 12, 95–106.

Sherr, C.J., 1996. Cancer cell cycles. Science 274, 1672–1677.

Sherr, C.J., 2000. The Pezcoller lecture: cancer cell cycles revisited. Cancer Res. 60, 3689–3695.

Shimamura, K., Hirano, S., McMahon, A.P., Takeichi, M., 1994. Wnt-1-dependent regulation of local E-cadherin and alpha N-catenin expression in the embryonic mouse brain. Development 120, 2225–2234.

Sive, H., Grainger, R., Harland, R., 2000. Early development of *Xenopus laevis*: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Stein, G.H., Beeson, M., Gordon, L., 1990. Failure to phosphorylate the retinoblastoma gene product in senescent human fibroblasts. Science 249, 666–669.

Szekely, L., Jiang, W.Q., Bulic-Jakus, F., Rosen, A., Ringertz, N., Klein, G., Wiman, K.G., 1992. Cell type and differentiation dependent heterogeneity in retinoblastoma protein expression in SCID mouse fetuses. Cell Growth Differ. 3, 149–156.

Thomas, D.M., Carty, S.A., Piscopo, D.M., Lee, J.S., Wang, W.F., Forrester, W. C., Hinds, P.W., 2001. The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. Mol. Cell 8, 303–316.

Vernon, A.E., Philpott, A., 2003. The developmental expression of cell cycle regulators in *Xenopus laevis*. Gene Expr. Patterns 3, 179–192.

Weinberg, R.A., 1995. The retinoblastoma protein and cell cycle control. Cell 81, 323–330.

Wildwater, M., Campilho, A., Perez-Perez, J.M., Heidstra, R., Blilou, I., Korthout, H., Chatterjee, J., Mariconti, L., Gruissem, W., Scheres, B., 2005. The retinoblastoma-related gene regulates stem cell maintenance in *Arabidopsis* roots. Cell 123, 1337–1349.

Williams, B.O., Schmitt, E.M., Remington, L., Bronson, R.T., Albert, D.M., Weinberg, R.A., Jacks, T., 1994. Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. EMBO J. 13, 4251–4259.

Wu, L., de Bruin, A., Saavedra, H.I., Starovic, M., Trimboli, A., Yang, Y., Opavska, J., Wilson, P., Thompson, J.C., Ostrowski, M.C., Rosol, T.J., Woollett, L.A., Weinstein, M., Cross, J.C., Robinson, M.L., Leone, G., 2003. Extra-embryonic function of Rb is essential for embryonic development and viability. Nature 421, 942–947.

Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J.D., Phillips, R.A., Gallie, B.L., 1996. pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. Genes Dev. 10, 3051–3064.